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(57) Abstract

Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino–acid corresponding to amino acid residue number 245 in *Photinus pyralis* which is such that the K_m for ATP of the luciferase is increased e.g. five–fold with respect to that of the corresponding non–mutated enzyme such that it is of the order of 500 μm – 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucloetides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real–time. Also disclosed are test–kits for *in vitro* assays.

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ENZYME ASSAYS

Technical Field

The present invention relates broadly to enzymes and methods suitable for assaying ATP. It further relates to specific applications for such assays.

Background Art

Intracellular ATP concentrations can vary 10-fold or more depending upon a cell's state of health or developmental stage. It is of great value to be able to measure fluctuations in intracellular ATP levels as a means of investigating e.g. the effects of drugs, toxins, hormones, environmental agents or disease on cells.

There is apparently at present no convenient method for analysing the concentration of ATP *in vivo*. For instance, in Dementieva et al (1996) Biochemistry (Moscow) Vol 61, No. 7., the intracellular concentration of ATP was measured in *E. coli* by calculating the total amount of ATP present using a recombinant luciferase, and dividing by an estimated total cell volume.

Such an indirect approach can at best produce only an estimate of the actual ATP concentration.

The measurement of ATP concentration in cells has also been performed using an *in vitro* coupled assay, such as that disclosed in the Sigma Diagnostic Kit Catalog No. 366, in which Phosphoglycerate kinase is used to convert 3-phosphoglycerate to 1,3 diphosphoglycerate in an [ATP]-dependent fashion. The 1,3 diphosphoglycerate is then converted to glyceraldehyde-3-P concommitantly with conversion of NADH to NAD, which can be monitored spectroscopically. The assay has a dynamic range up to 1 mM; the expected range is 380-620 μm when used with blood cells.

However it can be seen that, as with all coupled assays, the test is inevitably cumbersome to perform. Additionally it could not readily be adapted for *in vivo* use. It would thus be a contribution to the art to provide materials and methods which overcome some of the drawbacks of the prior art.

Disclosure of the Invention

In a first aspect of the invention there is provided a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme. Preferably the K_m is at least double that of the non-mutated enzyme, and more preferably at least around five, ten, or twenty times higher than that of the non-mutated enzyme.

Luciferases are, of course, already known in the art. In the presence of Mg^{2+}, luciferase (originally obtained from fireflies) catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2, pyrophosphate and light. This basic property (luciferin and ATP to produce light) is hereinafter referred to as 'luciferase activity'.

The term 'luciferase' as used in relation to the invention is intended to embrace all luciferases, or recombinant enzymes derived from luciferases which have luciferase activity. This explicitly includes recombinant mutant luciferases which have deletions, additions or substitutions to their amino acid structure provided that they retain luciferase activity. Such luciferases will typically have considerable homology (e.g. up to 70, 80, 90, or 99%) with wild-type enzymes. However the crucial technical feature of the luciferases of the present invention which distinguishes them from those of the prior art is that they have a mutation which causes an increase in the K_m for ATP of the luciferase as compared with that measured for a corresponding enzyme which differs only in it that it lacks that same mutation.

This increase K_m may be measured by the person of ordinary skill in the art by conventional enzyme assays, as described in more detail in the Examples below.

It should be noted that in the prior art, luciferase has sometimes been used as a marker for gene expression (*in vivo*) where its production in a cell is linked to a particular genetic control element. Luciferin is added exogenously and intracellular ATP concentrations, under almost all conditions, will be such that the enzyme is saturated. Thus the switching on of gene expression is signalled by light that is emitted in a quantitative manner according to the amount of active luciferase that is generated.

However it should be stressed that in the previously known systems it is generally the concentration of luciferase which is measured; this concentration is then correlated with a different event e.g. the efficiency of a promoter. Indeed it has, on occasions, been an object of the prior art teaching on luciferases to reduce the K_m for ATP (see e.g. WO 96/22376) which ensures that changes in the ambient [ATP] does not interfere with the assay.

Similarly the assay disclosed by Dementieva et al (1996) discussed above requires that all of the ATP be efficiently converted to light so that the total ATP present can be calculated. This approach requires a low K_m luciferase so that the enzyme operates at near maximal velocity until all the ATP is hydrolysed.

By making available luciferases which have an increased K_m compared with those already known in the art, the present inventors have for the first time opened up the possibility of using these enzymes to measure steady state ATP concentrations over range which was previously unsuitable. This is because, generally speaking, the relationship between enzyme velocity (V, as measured by light intensity) and substrate concentration (of ATP, where luciferin is in excess) is as follows:

$V = V_m . [ATP] / K_m + [ATP]$

It can therefore be seen that only when the K_m is greater than (or of a similar order as) the ambient [ATP] will there be a degree of proportionality between changes in [ATP] and changes in light intensity. Where the K_m is much less than the ambient [ATP], any changes in [ATP] will not tangibly effect the measured light intensity. Clearly the more sensitive the light detection is, the smaller the measurable changes in 'V' can be, and the smaller the K_m can be with respect to the [ATP] range being assessed.

For certain applications, e.g. *in vivo* measurements, it may be advantageous to have a luciferase wherein the K_m is of the order of between 400 μm to 1.4 mM e.g. 500 μm, 600 μm, 1 mM etc. However, as can be appreciated from the discussion above, the main criterion is that the K_m is not much less than the expected [ATP] range to be assessed, and the phrase 'of the order of' should be construed accordingly.

A particular expected **[ATP]** range which is important for physiological assays of blood cells is between 300 μm and 1 mM, or more particularly 380 μm and 620 μm, (cf. Sigma Diagnostic Kit, Catalog No. 366 discussed above). For other mammalian cells such as hepatocytes, the [ATP] range is 2.5 mM - 6 mM (see Dementieva et al (1996) discussed above. Use of the recombinant luciferases of the present invention for continuous assays in these ranges is particularly envisaged.

The disclosure of the present application makes such high K_m luciferases available for the first time. The prior art disclosures reveal only luciferases having a K_m of between 60 μm and 150 μm, which would be saturated in these ranges.

It is also advantageous, as with all enzymes used in assays, that the mutant enzyme retains sufficient activity (i.e. a high maximum turnover number, giving a high V_m) such that practical concentrations of enzyme can give detectable results.

Preferably the activity for ATP of the mutant is at least 5-100% of that of the corresponding wild-type; however reduced-activity as a result of the high K_m mutation can, if necessary, be compensated for by using more enzyme or more sensitive detection if required.

In one embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

It should be noted that the sequences of a number of luciferases from different sources have already been published in the literature, see e.g. WO 95/25798 for *P pyralis*; EP 0 524 448 for *Luciola cruciata* and *Luciola lateralis*. Other known *luc* genes include *Luciola mingrelica*, and *Lampyris noctiluca* (see Newby et al (1996) Biochemical J 313: 761-767.)

Whether an amino-acid in a luciferase 'corresponds' to number 245 in *P pyralis* (which is His in the wild-type, non-mutated enzyme) can be established by the person of ordinary skill in the art without difficulty as follows: the sequence of the luciferase is established (either from the literature or by sequencing); the sequence is aligned with *P pyralis*, for instance using commercially available software (e.g. "Bestfit" from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) or manually such as to demonstrate maximal homology and align conserved amino acids; the amino acid corresponding to number 245 in *P pyralis* is identified. An example of this is shown below using *L cruciata* - the corresponding amino acid in that case is number 247.

Once identified a mutant can be prepared e.g. by site directed mutagenesis by methods commonly used in the art and exemplified below.

Preferably corresponding amino-acid is substituted for an uncharged amino acid, for instance nonpolar (e.g. Ala) or uncharged polar (e.g. Asn, or Gln):

CLASS	EXAMPLES OF AMINO ACID
Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic:	Asp, Glu
Basic:	Lys, Arg, His

It should be noted that WO 95/18853 (PROMEGA) lists a large number (over 80) of *Pyrophorus plagiophtalamus* mutants which are reported to have altered spectral properties. However the K_m for ATP of the mutants is not reported, nor indeed discussed at any point in the application.

In another embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 318 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme. Correspondence may be assessed as above; preferably the amino acid (Ser in the wild type) is substituted for a bulkier one (e.g. Thr).

In preferred forms the mutant luciferases of the present invention incorporate one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light. Some suitable mutations are already known to those skilled in the art; see e.g. WO 95/25798 and WO 96/22376 and EP 0 524 448 for thermostability improving mutations (e.g. at positions corresponding to 354 and 215 of *P pyralis*).

Preferably the mutation causing the increased K_m itself improves one or more of these properties, particularly thermostability. It should be noted that an enhanced stability at around 37°C is especially advantageous for enzymes which are to be employed *in vivo*.

In a further embodiment the luciferases may be in the form of fusion proteins or incorporate polypeptide extensions. This may improve the ease by which they can be produced, localised *in vivo* or extracted and purified.

In a second aspect of the invention there is disclosed a recombinant polynucleotide encoding a mutant luciferase of the present invention, as described above.

In a third aspect there are disclosed vectors comprising a polynucleotide of the second aspect. For instance vectors further comprising a replication element which permits replication of the vector in a suitable host cell and/or a promoter element which permits expression of said polynucleotide in a suitable host cell. The promoter may be a constitutive promoter. Optionally the promoter element may be tissue- or organ-specific.

In a fourth aspect there is disclosed a host cell containing, or transformed with, a vector of the third aspect.

Optionally the host cell of the fourth aspect may express one or more further luciferases which have a lower K_m for ATP than those of the present invention, and possibly emit light of a different wavelength, such as to extend the useful range of any assay, and/or allow the use of a ratiometric assay i.e. one in which the activity of the high K_m mutant is compared with that of a further luciferase. The further luciferases may be recombinant non-mutant luciferases or recombinant mutant luciferases having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme (see e.g. WO 96/22376 .

Coloured mutants are disclosed in WO 95/18853 and in Ohmiya et al (1996) FEBS Letters 384: 83-86.

In a fifth aspect there is disclosed a process for producing a luciferase of the present invention comprising culturing a host cell as described in the fourth aspect.

In a sixth aspect there is disclosed a single cell organism consisting of a host cell as described above, or a multicellular organ or organism comprising it. The use of e.g. transgenic higher animals in which the luciferases of the present invention are expressed could allow *in vivo* study of [ATP] in different types of cell or tissue as described in more detail below. In particular, as ATP is present in virtually all living cells, any type of cell into which luciferase could be cloned, from bacterial to plant or animal, could be studied through the measurement of ATP changes.

Thus in a seventh aspect of invention there is disclosed a method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as described above.

Preferably the method comprises the following steps(a) the luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

The measurement in step (b) may be compared with a control value such as minimise base-line errors.

The assay can be *in vitro* or *in vivo*.

More preferably the material itself is a cell, in to which the luciferase is introduced e.g. by transforming the cell with a vector as described above. Alternatively the luciferase may be introduced into the cell by direct injection.

Equally the material measured may be part of a synapse i.e. the ATP is neurotransmitter.

Generally the assay will be most useful for real-time analysis (on a time-scale of seconds e.g. using a CCD camera, photomultiplier or photodiode) of events initiated by particular stimuli (e.g. addition of an active agent to the material). In this case the assay can monitor changes in [ATP] concentration over a relatively short time-scale. Such measurement will not, therefore, be greatly affected by longer time-scale events, such as changes in the concentration of luciferase in the system. These changes can be correlated with cellular events e.g. tissue necrosis may be associated with falling [ATP], fatigue in muscle likewise. Such continuous assays have hitherto not been possible.

Other possible applications include measuring the effect of drug treatments on various tissues; toxins and uncoupling agents on oxidative phosphorylation; bacterial infection; metabolic processes and stress (e.g. obesity and exercise); studies of brain activity (e.g. memory function and mental disorders) etc.

If appropriate the [ATP] can be measured periodically (rather than constantly) using photographic film.

Essentially the monitoring can be done in ways analogous to those already used in the art for other applications e.g. for the photonic detection of bacterial pathogens in living hosts disclosed by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In that paper a Hamamatsu intensified CCD camera was used to visualise *Salmonella Typhimurium* expressing luciferase during infection of a mouse. Equally a system equivalent to PET (positron emission tomography - as used in brain scans) could be used to achieve precise localisation of luciferase-generated light to allow the metabolism of specific body regions to be ascertained.

Generally speaking it will be necessary to introduce luciferin into the system being studied. By 'luciferin' is meant any co-factor which has luciferin activity i.e. can be used in conjunction with luciferase to cause light to be emitted in the presence of ATP. The manner by which this is introduced in to the system will depend on the system itself. For instance where animal cells are being studied, luciferin may be introduced by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part. Similarly when the system being studied is one or more plant cells, the luciferin may simply be introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

In a final aspect of the invention there is disclosed a test kit comprising a luciferase discussed above and further comprising one or more of the following(a) a buffer or dry materials for preparing a buffer; (b) ATP standards; (c) luciferin; (d) Dithiothreitol (e) instructions for carrying out an ATP assay.

The invention will now be further described with reference to the following none-limiting Figures, Sequence Listings and Examples.

Figures

Fig 1. shows plasmid pPW601a as described in Example 1.

Fig 2. for mutant H245A, (a) shows the plot of V against [ATP] and (b) shows 1/V against 1/[ATP] as described in Example 1.

Fig 3. shows a sequence comparison of one region of *P pyralis* (Pp) and the corresponding region of *L cruciata* (Lc) as described in Example 2.

Fig 4 is a graph showing light emission versus ATP concentration for mutant H245N.

Fig 5 . shows the effect of the addition of nutrient broth to luciferase-expressing E. coli cells pre-charged with Luciferin as described in Example 6.

Fig. 6 shows Seq ID No. 1 which is the nucleotide sequence of the wild type *luc* gene from *P pyralis*.

Fig. 7 shows Seq ID Nos. 2-5 which are the primers used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4) and the equivalent wild-type sequence (Seq ID No 5).

Fig. 8 shows Seq ID No. 6 which is the amino acid sequence of a high K_m mutant H245Q of the present invention, wherein amino acid 245 has been changed to Gln.

Examples

EXAMPLE 1: PRODUCTION OF RECOMBINANT HIGH K_M MUTANT LUCIFERASE

Except where otherwise stated, the methods employed were as those used by White et al (1996) Biochemical Journal 319: 342-350, which is concerned with thermostable mutants.

STARTING MATERIALS: Mutants were generated by site directed mutagenesis of the plasmid pPW601a (Fig 1) comprising the luciferase gene, *luc*, from *P pyralis*. The wild type *luc* gene from *P pyralis* is shown at Seq ID No. 1. Plasmid pPW601a was created by cloning the *luc* gene *Bam*HI/*Sst*I fragment from pGEM-*luc* (available from Promega) into pDR540 (available from Pharmacia). The unique *Xho*I site in the polylinker of the plasmid was removed to simplify the following procedures.

SITE DIRECTED MUTAGENESIS: Three mutagenic oligonucleotides were used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4). The equivalent wild-type sequence is shown at Seq ID No. 5. The oligonucleotides also introduced a silent mutation which destroys a unique Xmn I site in the *luc* gene - this did not result in an amino acid

substitution but facilitated mutant selection. The mutagenesis was carried out in accordance with the kit instructions of kit supplied by Clontech laboratories Inc, Palo Alto, California USA.

The amino acid sequence of H245Q is shown in Seq. ID No. 6.

ISOLATION OF PLASMID DNA & TRANSFORMATION: this was carried out by the method of Brinboim & Doly (1979) Nucleic Acids Research 7: 1513.

CELL CULTURE AND EXTRACTION: *E. coli* JM109 transformants were grown to an OD_{600} = 1.0. Aliquots of cells expressing mutant luciferases from plasmid pPW601a, were subjected to lysis as described in the Promega technical bulletin and the lysed extracts were then stored on ice prior to assay.

ASSAY OF Km OF MUTANT LUCIFERASES: luciferase assays were performed at 21°C using 100 µl of assay buffer (20 mM Tricine pH 7.8 containing 2.0 mM $MgSO_4$, 0.1 mM EDTA, 33mM dithiotheitol, 470 µM D-luciferin and ATP in the concentration range 6.25 - 800 µM). Each assay contained 5-10 µl of crude cell extract.

The plots of V against [ATP] and 1/V against 1/[ATP] for mutant H245A are shown in Fig. 2. Such plots kan be used to determine the K_m.

The results of each mutation and the recombinant Wild Type are shown in Table 1:

Table 1

Luciferase	K_m MgATP (μM)
r Wild Type	66
H245A	442
H245N	623
H245Q	1340
A215L*	65

* A215L is a thermostable mutant in which amino acid 215 is substituted with lysine (see WO 96/22376 - SECRETARY OF STATE FOR DEFENCE).

ASSAY OF THERMOSTABILITY OF MUTANT LUCIFERASES: the thermostability of H245N & H245Q was also tested, as compared with mutant A215L and the wild-type. Lysed crude extracts of cells containing luciferase activity were incubated at 37°C for set time periods. The thermostability of the mutant H245A was found to be very similar to that of the recombinant wild-type. The results are shown in Table 2:

Table 2

Enzyme	Remaining activity %			
	0	2	4	8 minutes
r Wild Type	100	64.8	36.6	26.6
A215L	100	101	88	84
H245N	100	96	61	46
H245Q	100	103	78.6	51.5

PURIFICATION: luciferases, e.g. incorporating the H245Q mutation, may be purified as described in White et al (1996) [supra]. Briefly, the cell lysates are centrifuged at 30000 g for 30 mins and the supernatant is fractionated with ammonium sulphate (30-55%). This fraction is resuspended and desalted. The desalted material was passed through a hydroxyapatite column and eluted with 10-200 mM sodium phosphate containing dithiothreitol. The luciferase containing eluant is dialysed and applied to a Mono Q anion-exchange column. The enzyme can be eluted with 0 to 500 mM NaCl.

EXAMPLE 2: IDENTIFICATION OF CORRESPONDING HIGH K_M MUTANTS

Fig 3. shows a sequence comparison of one region of *P pyralis* and the corresponding region of *L cruciata* as describe din Example 2. In this case it can be seen that amino acid 245 corresponds to 247.

EXAMPLE 3: EXPRESSION OF MUTANT LUCIFERASE IN MAMMALS

This can be achieved by methods analogous to those disclosed by Liu et al (1997) Nature Biotechnology 15: 167-173. In this method cationic liposomes are used to deliver plasmid DNA containing luciferase a gene to mice.

EXAMPLE 4: AN *IN VIVO* ATP ASSAY IN MAMMALS

This can be carried out by methods analogous to those used by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In this method luciferase expression in *S typhimurium* in mice is monitored using a CCD camera.

EXAMPLE 5: A KIT FOR AN *IN VITRO* ATP ASSAY

This may be provided as follows: luciferase H245Q; buffer; or dry materials for preparing a buffer; ATP for standards; luciferin; and instructions for carrying out an ATP assay.

EXAMPLE 6 : Assay for determining cell behaviour

Using a luciferase assay as described in Example 1, a plot of the photon count versus the ATP concentration was prepared for the H245N mutant. The results are shown in Figure 4.

In order to demonstrate how the enzyme of the invention can be used in studying cellular behaviour, a sample of recombinant E.coli cells which expressed the H245N mutant luciferase were rendered dormant by exhaustion of nutrients. The cells were charged with luciferin by 10 minutes immersion in p.H. 5.0 citrate buffer containing 1mM luciferin. They were then centrifugally washed, resuspended in 1ml Nutrient Broth and the luminescence monitored. The results are shown in Figure 5.

Using the mutant luciferase of the invention, the revival and growth of functional cells could be monitored.

CLAIMS

1. A recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme.

2. A luciferase as claimed in claim 1 wherein the K_m is at least double that of the non-mutated enzyme.

3. A luciferase as claimed in claim 2 wherein the K_m is at least five times higher than that of the non-mutated enzyme.

4. A luciferase as claimed in claim 1 wherein the K_m is of the order of 500 μm.

5. A luciferase as claimed in claim 1 wherein the K_m is of the order of 1 mM.

6. A luciferase as claimed in any one of the preceding claims having a V_m for ATP which is at least 5-100% of that of the corresponding wild-type.

7. A luciferase as claimed in any one of the preceding claims wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

8. A luciferase as claimed in claim 7 wherein the amino-acid has been substituted for an uncharged amino acid.

9. A luciferase as claimed in claim 8 wherein the amino-acid has been substituted for Ala, Asn, or Gln.

10. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Photinus pyralis* and wherein amino acid residue number 245 has been substituted.

11. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Luciola cruciata* and wherein amino acid residue number 247 has been substituted.

12. A luciferase as claimed in any one of the preceding claims incorporating one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light.

13. A fusion protein comprising a luciferase as claimed in any one of the preceding claims.

14. A recombinant polynucleotide encoding a luciferase as claimed in any one of the claims 1 to 12.

15. A replication vector comprising a polynucleotide as claimed in claim 14 further comprising a replication element which permits replication of the vector in a suitable host cell.

16. An expression vector comprising a polynucleotide as claimed in claim 14 further comprising a promoter element which permits expression of said polynucleotide in a suitable host cell.

17. A vector as claimed in claim 16 wherein the promoter element is tissue or organ specific.

18. A host cell containing a vector as claimed in any one of claims 15 to 17.

19. A host cell transformed with a vector as claimed in any one of claims 15 to 17.

20. A host cell as claimed in claim 19 which also expresses a second luciferase having a lower K_m for ATP.

21. A host cell as claimed in claim 20 wherein the second luciferase is selected from: (a) a recombinant non-mutant

luciferase; (b) a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme.

22. A process for producing a luciferase comprising culturing a host cell as claimed in any one of claims 19 to 21.

23. A host organism consisting of or comprising a host cell as claimed in any one of claims 19 to 21.

24. Use of a recombinant luciferase as claimed in any one of claims 1 to 12 for assaying the amount of ATP in a material, wherein the concentration of the ATP is expected to be between 300 μm and 6 mM.

25. A method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as claimed in any one of claims 1 to 12.

26. A method as claimed in claim 25 wherein (a) the luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

27. A method as claimed in claim 26 wherein the measurement in step (b) is compared with a control value.

28. A method as claimed in claim 26 wherein the measurement in step (b) is made in real-time.

29. A method as claimed in any one of claims 25 to 28 wherein the material measured forms part of a synapse.

30. A method as claimed in any one of claims 25 to 28 wherein the material is a cell and the luciferase is introduced into the cell.

31. A method as claimed in claim 30 wherein the luciferase is introduced into the cell by transforming the cell with a vector as claimed in any one of claims 15 to 17.

32. A method as claimed in claim 30 or claim 31 wherein the luciferin is introduced into the cell by direct injection.

33. A method as claimed in claim 30 or claim 31 wherein the cell is an animal cell and the luciferin is introduced into the cell by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part.

34. A method as claimed in claim 30 or claim 31 wherein the cell is a plant cell and the luciferin is introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

35. A test kit comprising a luciferase as claimed in any one of claims 1 to 12 and further comprising one or more of the following(a) a buffer or dry materials for preparing a buffer; (b) two or more measured portions of ATP suitable for preparing standard solutions; (c) luciferin; (d) instructions for carrying out an ATP assay.

Fig.1.

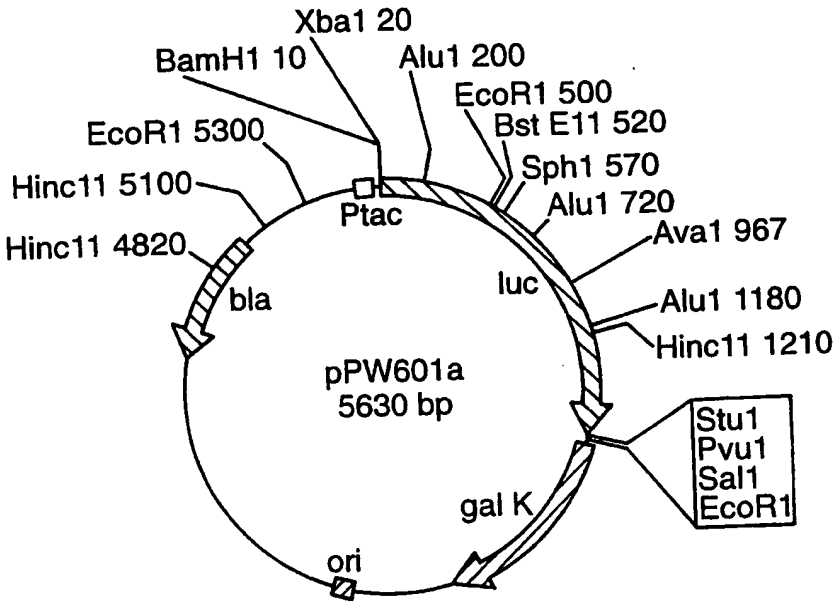
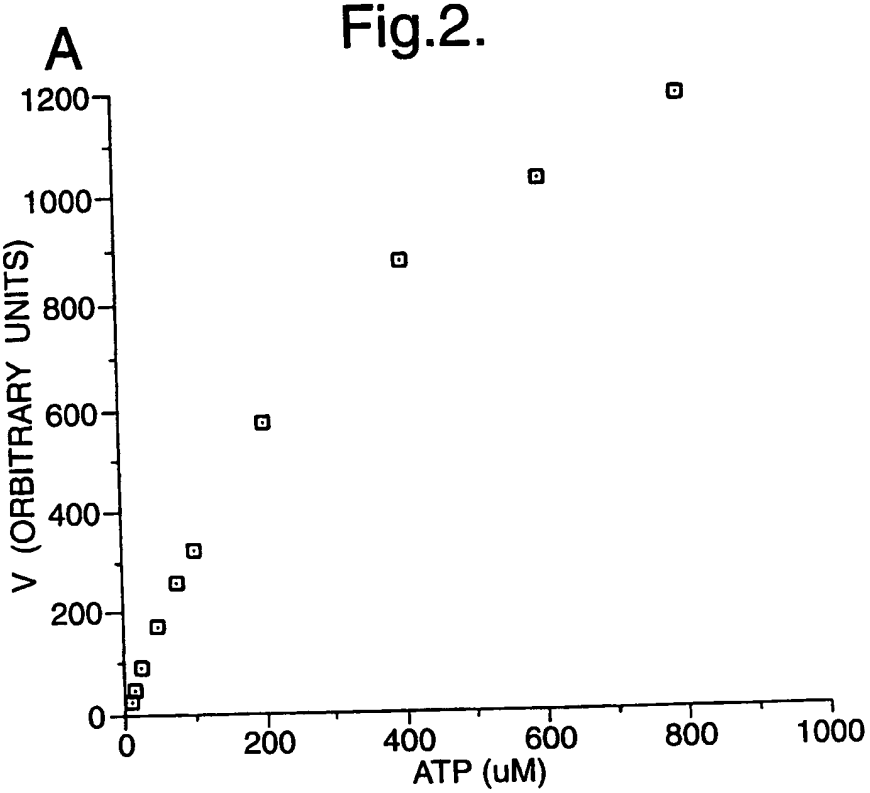

Xba1 20
BamH1 10
Alu1 200
EcoR1 500
Bst E11 520
EcoR1 5300
Sph1 570
Hinc11 5100
Alu1 720
Ptac
Ava1 967
Hinc11 4820
luc
bla
Alu1 1180
Hinc11 1210
pPW601a
5630 bp
Stu1
Pvu1
Sal1
EcoR1
gal K
ori

Fig.2.


A
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1000
800
600
400
200
0
V (ORBITRARY UNITS)
0
200
400
600
800
1000
ATP (uM)

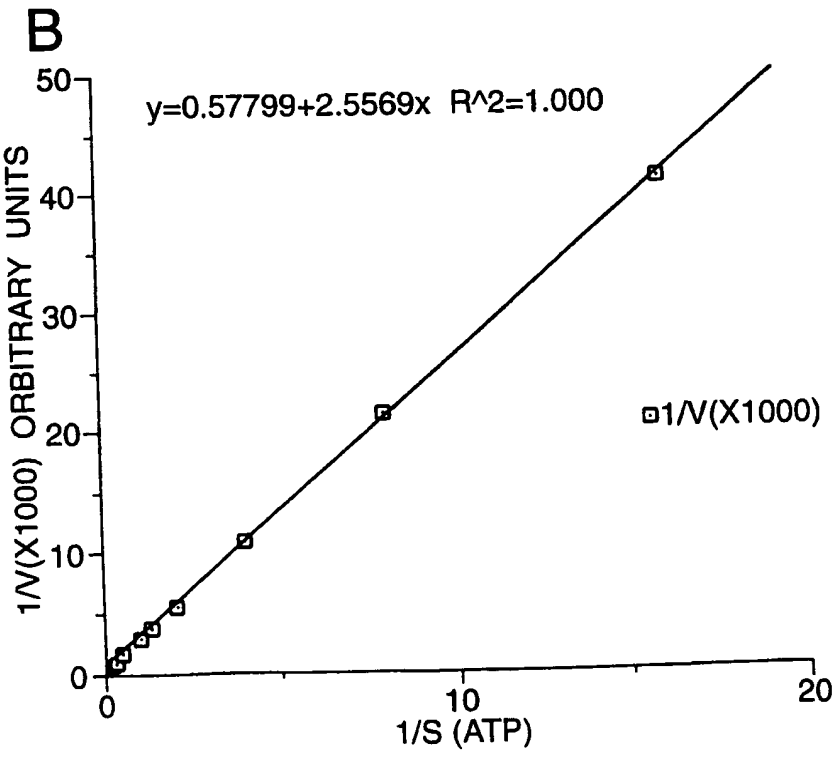
B
y=0.57799+2.5569x R^2=1.000
50
40
30
20
10
0
1/V(X1000) ORBITRARY UNITS
1/V(X1000)
0
10
20
1/S (ATP)

Fig.3.

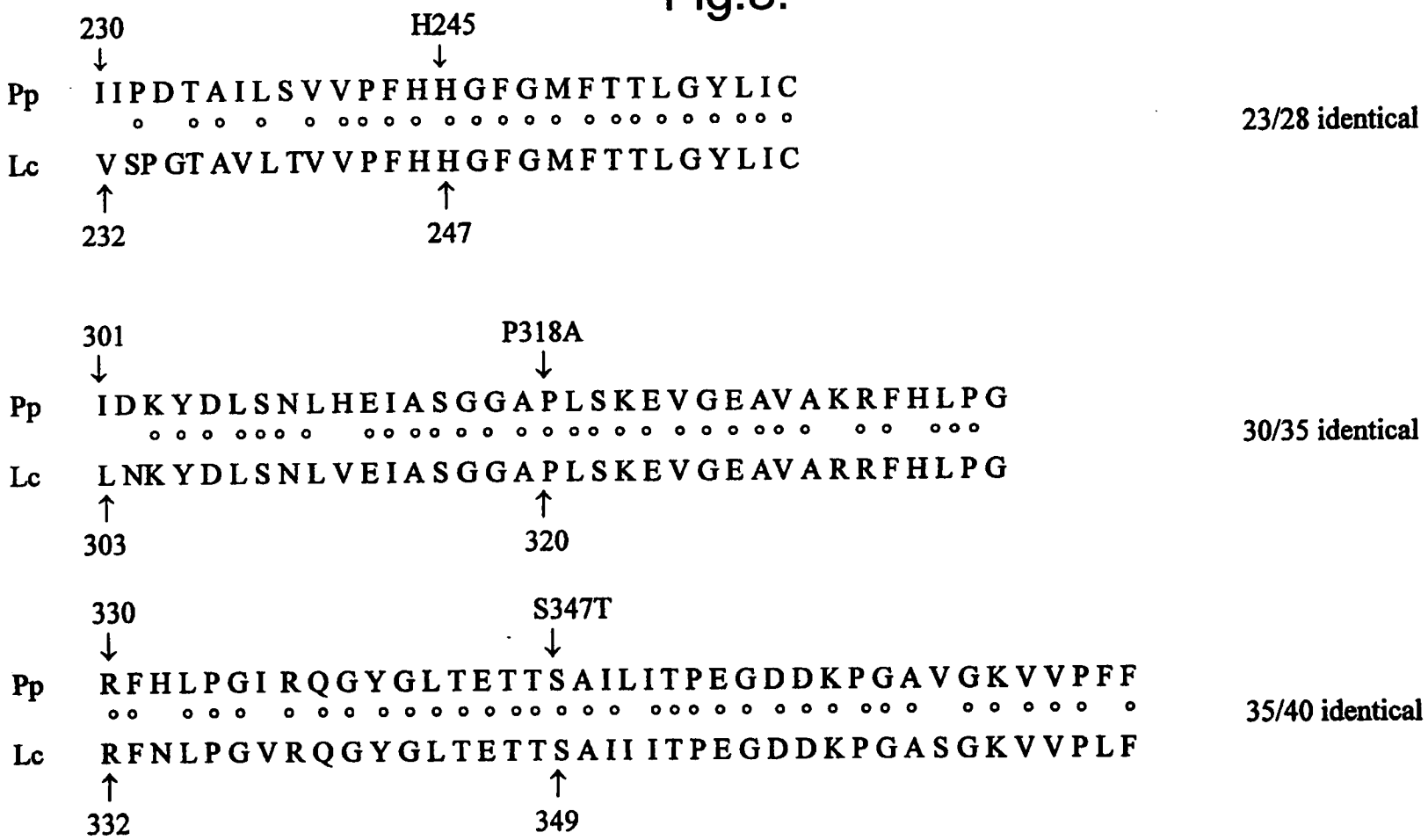
230
H245
Pp IIPDTAILSVVPFHHGFGMFTTLGYLIC
23/28 identical
Lc VSPGTAVLTVVPFHHGFGMFTTLGYLIC
232
247
301
P318A
Pp IDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPG
30/35 identical
Lc LNKYDLSNLVEIASGGAPLSKEVGEAVARRFHLPG
303
320
330
S347T
Pp RFHLPGIRQGYGLTETTSAILITPEGDDKPGAVGKVVPFF
35/40 identical
Lc RFNLPGVRQGYGLTETTSAII ITPEGDDKPGASGKVVPLF
332
349

Fig.4.

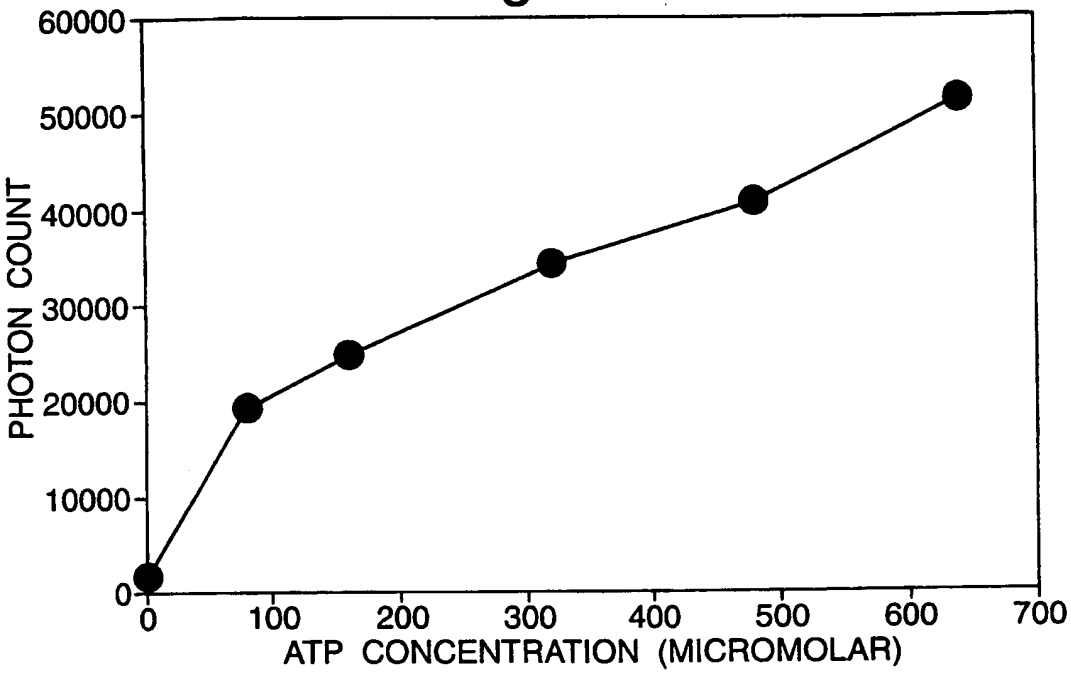
60000
50000
40000
30000
20000
10000
0
PHOTON COUNT
0
100
200
300
400
500
600
700
ATP CONCENTRATION (MICROMOLAR)

Fig.5.

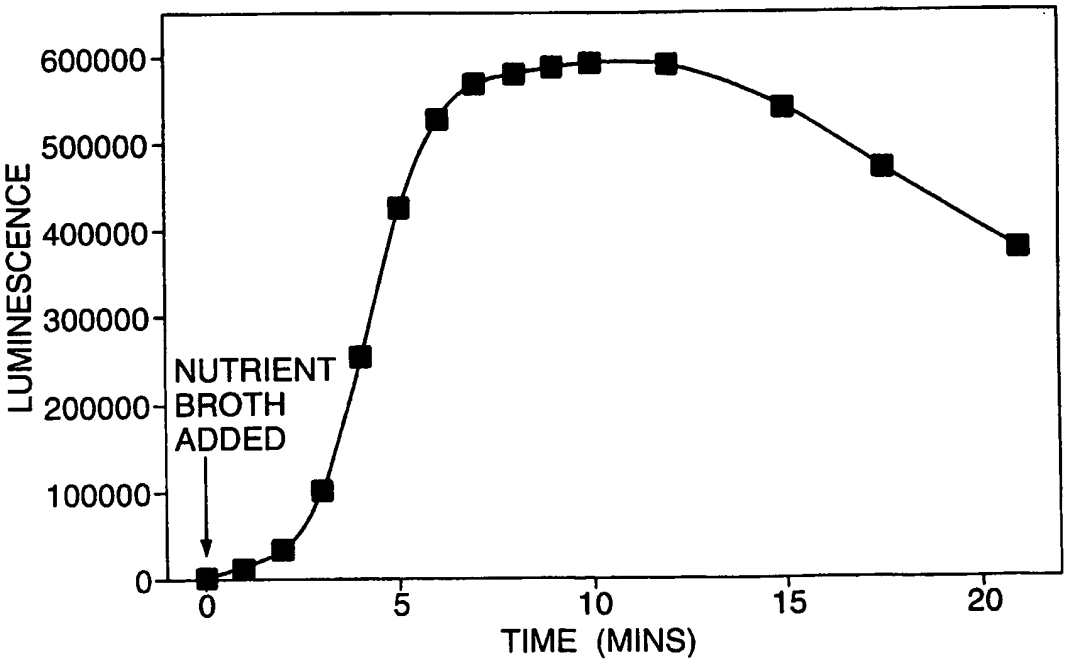
600000
500000
400000
300000
200000
100000
0
LUMINESCENCE
NUTRIENT
BROTH
ADDED
0
5
10
15
20
TIME (MINS)

Fig.6.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1722 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 4..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAAATGGAAG ACGCCAAAAA CATAAAGAAA GGCCCGGCGC CATTCTATCC TCTAGAGGAT 60

GGAACCGCTG GAGAGCAACT GCATAAGGCT ATGAAGAGAT ACGCCCTGGT TCCTGGAACA 120

ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG 180

TCCGTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC 240

GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA 300

GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC 360

ATTTCGCAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG 420

CAAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG 480

GGATTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC 540

GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT 600

GGATCTACTG GGTTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC 660

TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT 720

Fig.6 (Cont).

GTTGTTCCAT TCCATCACGG TTTTGGAATG TTTACTACAC TCGGATATTT GATATGTGGA 780

TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT 840

TACAAAATTC AAAGTGCGTT GCTAGTACCA ACCCTATTTT CATTCTTCGC CAAAAGCACT 900

CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCTGGGGG CGCACCTCTT 960

TCGAAAGAAG TCGGGGAAGC GGTTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA 1020

TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG 1080

GGCGCGGTCG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG 1140

AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC 1200

GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT 1260

TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG 1320

TCTTTAATTA AATACAAAGG ATATCAGGTG GCCCCCGCTG AATTGGAATC GATATTGTTA 1380

CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA 1440

CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG 1500

GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG 1560

GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC 1620

ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC 1680

GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA 1722

Fig.7.

MUTAGENIC OLIGONUCLEOTIDES

Sequence ID No. 2

H245A:

736 737 744
↓↓ ↓
5' - GTT GTT CCA TTC CAT gcC GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 3

H245Q:

738 744
↓ ↓
5' - GTT GTT CCA TTC CAT CAg GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 4

H245N:

736 744
↓ ↓
5' - GTT GTT CCA TTC CAT aAC GGT TTc GGA ATG TTT AC-3'

Sequence ID No. 5

WILD TYPE SEQUENCE:

721 H245 nt 753
↓ | | ↓
5' - GTT GTT CCA TTC CAT CAC GGT TTT GGA ATG TTT AC-3'

nt numbering is from luc gene sequence, CAA .... nt 1

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 550 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

Fig.8.

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Photinus pyralis

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1 5 10 15

Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20 25 30

Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35 40 45

Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50 55 60

Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65 70 75 80

Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85 90 95

Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100 105 110

Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115 120 125

Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130 135 140

Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145 150 155 160

Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165 170 175

Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180 185 190

Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195 200 205

Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210 215 220

Fig.8 (Cont).

Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
225 230 235 240

Val Pro Phe His Gln Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
245 250 255

Ile Cys Gly Phe Arg Val Val Leu Met Tyr Arg Phe Glu Glu Glu Leu
260 265 270

Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
290 295 300

Asp Leu Ser Asn Leu His Glu Ile Ala Ser Gly Gly Ala Pro Leu Ser
305 310 315 320

Lys Glu Val Gly Glu Ala Val Ala Lys Arg Phe His Leu Pro Gly Ile
325 330 335

Arg Gln Gly Tyr Gly Leu Thr Glu Thr Thr Ser Ala Ile Leu Ile Thr
340 345 350

Pro Glu Gly Asp Asp Lys Pro Gly Ala Val Gly Lys Val Val Pro Phe
355 360 365

Phe Glu Ala Lys Val Val Asp Leu Asp Thr Gly Lys Thr Leu Gly Val
370 375 380

Asn Gln Arg Gly Glu Leu Cys Val Arg Gly Pro Met Ile Met Ser Gly
385 390 395 400

Tyr Val Asn Asn Pro Glu Ala Thr Asn Ala Leu Ile Asp Lys Asp Gly
405 410 415

Trp Leu His Ser Gly Asp Ile Ala Tyr Trp Asp Glu Asp Glu His Phe
420 425 430

Phe Ile Val Asp Arg Leu Lys Ser Leu Ile Lys Tyr Lys Gly Tyr Gln
435 440 445

Val Ala Pro Ala Glu Leu Glu Ser Ile Leu Leu Gln His Pro Asn Ile
450 455 460

Phe Asp Ala Gly Val Ala Gly Leu Pro Asp Asp Asp Ala Gly Glu Leu
465 470 475 480

Pro Ala Ala Val Val Val Leu Glu His Gly Lys Thr Met Thr Glu Lys
485 490 495

Glu Ile Val Asp Tyr Val Ala Ser Gln Val Thr Thr Ala Lys Lys Leu
500 505 510

Arg Gly Gly Val Val Phe Val Asp Glu Val Pro Lys Gly Leu Thr Gly
515 520 525

Lys Leu Asp Ala Arg Lys Ile Arg Glu Ile Leu Ile Lys Ala Lys Lys
530 535 540

Gly Gly Lys Ser Lys Leu
545 550